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Original article

Effect of linseed diet and hazelnut skin supplementation on oxidative status and blood serum metabolites in rabbits

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Summary. Rabbits in groups of eight were fed for nine weeks with diets containing linseed, rich in polyunsaturated fatty acids (LS), linseed plus hazelnut skins, with antioxidant function (LS+HS), or palm oil, rich in saturated fatty acids (PO). The aim of this work was to compare the effects of aforementioned diets on serum biochemical parameters related to nutritional and inflammatory status, immune function and oxidative balance [triglycerides, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lysozyme, reactive oxygen species and antioxidant power, respectively]. At the end of the study redox imbalance (with a significant increase in oxidative stress and decrease in antioxidant defenses) was greatest in rabbits fed the LS diet (without additional antioxidant protection) compared to the other two groups ($P<0.05$). Also the blood value of lysozyme, an immune parameter linked to inflammatory phenomena, was greater in the LS compared to the other two groups ($P <0.05$). ALT remained unchanged in all three groups, while a significant increase in AST, triglycerides, and cholesterol was noted in the group fed the PO diet as compared to the other two groups ($P<0.05$). In conclusion, the most favorable and healthy values were found in rabbits fed with diets containing linseed and supplemented with hazelnut skins (LS+HS group).

Key words: linseed, hazelnut skins, palm oil, rabbit, oxidative status, health, welfare

Introduction

In recent years, substantial effort has been devoted to the safeguard of livestock welfare to guarantee optimal living and growth conditions and to reduce the occurrence of multifactorial diseases which decrease zootechnical productivity (1,2).

Biochemical screening for assessing livestock growth and welfare is usually performed to monitor physiological or pathological responses to changes in rearing conditions (3). This has had an impact on consumer awareness of animal welfare and animal-friendly products. Among others, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides and cholesterol represent the most screened indices, which better reflect nutritional status of rabbits (4). ALT and AST are clinically significant aminotransferases. Both are markers of liver function, organ particularly devoted to nutrients and xenobiotic transformation and protein synthesis; however, ALT is considered more liver-specific than AST. Those markers are commonly used in humans and animals to indagate inflammatory and degenerative conditions of the liver, such as non-alcoholic fatty liver disease (NAFLD), which is linked to improper nutrition. According to (5), ALT increase positively correlates with severity of liver damage and also represents an excellent screening test to detect subclinical NAFLD. Risk factors for NAFLD include obesity, diabetes and hyperlipidemia. Elevated ALT may be also a component of the meta-

bolic syndrome, the hallmark of which is insulin resistance, manifested by hyperglycemia, hyperlipidemia, abdominal obesity and hypertension in humans. Given the above and considering physiological similarities existing between humans and rabbits, triglycerides and cholesterol levels could be monitored as well in farm animals, in order to precisely depict their metabolic and nutritional status during rearing changes. Moreover; increased ALT, triglycerides and cholesterol levels have been associated with deaths from liver disease and all-cause mortality in some human studies (5).

Determination of the oxidative plasmatic status and immune function in farm animals could also have implications of veterinary interest. For instance, suboptimal feeding or management strategies on the farm can be identified, and appropriate interventions subsequently adopted to create adequate conditions for livestock. Oxidative stress, for example, is implicated in the pathogenesis of various different illnesses, including those associated with animal production, reproduction and welfare in rabbits and other farm animals (6,7). Its assessment is carried out to determine whether the animal is able to cope with stressful environmental stimuli by maintaining a homeostatic condition (8). Under circumstances of pathological stress, an organism's adaptive response is frequently inadequate, leading to the overproduction of free radicals that results in oxidative stress (9,10).

To monitor immune function, serum lysozyme determination could be carried out in addition to aforementioned markers. Lysozyme is an antimicrobial enzyme produced by animals that forms part of the innate immune system.

Nutritional interventions, however, could represent a "double-edge sword", potentially exacerbating a pre-existing condition of metabolic stress, redox imbalance and immune-depression. For instance, consumer demand for animal products with a higher content of n-3 polyunsaturated fatty acids (PUFA), which is beneficial for animal and human health, has increased during the last decades (11). Furthermore, n-3 PUFA enriched with antioxidants provides for healthier diets for broilers (12,13), pigs (14) and rabbits (15), enhanced welfare during breeding, and ultimately healthier products of animal origin. In contrast, diets high in saturated fatty acids (SFA) have a negative effect on animals fed diets enriched with animal fat (increased serum cholesterol) and on the characteristics of their meat quality (increased atherogenic and thrombogenic indexes) (13).

A good dietary source of PUFAs is linseed, which is a potential commercial source of α-linolenic acid. Its inclusion in diets has been investigated in previous studies on rabbits as a way to produce n-3 PUFA enriched meats for functional food of animal origin (4, 16-22). Since the early 20th century, the addition to livestock diet of linseed in the form of boiled ground seeds or pellets to increase seed digestibility (23) has been shown to enhance immune function and cardiovascular function, blood pressure, cholesterol, and triglyceride levels in animals and humans (11). The n-3 PUFA easily undergoes peroxidative damage and the higher content of n-3 PUFA in linseed could make supplemented diets more susceptible to lipid oxidation. Since this could also trigger an increase in free radical production, the incorporation of antioxidants in diets rich in PUFAs to prevent lipid oxidation and its harmful cascade represents a crucial point when adopting nutritional strategies in livestock to obtain functional foods while preserving animal welfare (24-27). (28) studied the endocrine response, reproductive long-term effects and fatty acid profile of rabbit does fed diets supplemented with n-3 PUFA that tended to improve endocrine function, while (29) evaluated the effects of n-3 PUFA supplemented diet on the metabolic and endocrine response of rabbit does and their offspring and found that this supplementation reduced cholesterol concentration of does during lactation, but increased the oxidative stress in both does and kits.

In the present study, we tested the addition of hazelnut (*Corylus avellana* L.) skins (HS) to diet for their natural antioxidant properties. The use of HS as a potential source of natural antioxidant and functional food ingredient is gaining attention. The hazelnut tree belongs to the *Betulaceae* family and has worldwide distribution. Its positive effects on human and animal health have been documented regarding the antioxidant activity and phenolic constituents of HS (30-33). It follows then that extracts of natural antioxidants from HS could find potential use as nutraceuticals and dietary supplements for animals. Furthermore, in geographical areas where HS are an abundant and cheap by-product of the food industry their inclusion in live-

stock diets could be advantageous for both nutritional and ecological reasons.

Diets rich in PUFAs are used and recommended in livestock to increase the intake of these lipid components in products of animal origin; however, if not adequately supplemented with antioxidants, these diets can lead to oxidative and inflammatory stress in the animal due to increased susceptibility to lipid peroxidation as compared to SFA. While these latter are more stable against oxidation, they are nutritionally inappropriate for the reasons mentioned above. Based on this evidence, the aim of the present study was to evaluate the effects of linseed and HS supplementation on serum biochemical parameters ALT, AST, triglycerides and cholesterol, oxidative plasmatic status (as measured by d-ROMs test and OXY test) and immune function (lysozyme) in young rabbits, as serum indices in relation to their welfare during rearing. The aforementioned screening represents a minimally invasive, cost- and time-saving approach for the evaluation of rabbits' health and wellness.

Material and Methods

Animals and Feeding

Rabbits were handled according to the principles stated in D.Lvo 26/2014 (implementation of EC directive 63/2010) regarding the protection of animals used for experimental and other scientific purposes.

The study was carried out at the experimental rabbitry at Castenuovo Don Bosco (AT) supervised by the Department of Veterinary Science, University of Torino. A total of 24 grey Carmagnola female rabbit [mean age 6 months; mean body weight 3901±9 g at the start (day 1) and 3907±14 g at the end of the experimental period (day 63)] were randomly divided into 3 groups of 8 rabbits per group. Selecting only female animals was an arbitrary decision to reduce gender variability of the parameters to assess animal welfare.

The animals were housed individually in wire cages (50 cm × 60 cm × 35 cm height) and had free access to clean drinking water. The temperature and photoperiod in the rabbitry were 22±2°C and 16L:8D, respectively. The rabbits were fed with diets enriched with: linseed at 5% (LS), linseed at 5% plus hazelnut skins at 1.5% (LS+HS), or palm oil at 1.8% (PO), respectively. Diet composition in term of levels of PUFA/SFA and antioxidants represented the only experimental variable among groups, able to interfere with wellness, redox status and immune function. The diets were formulated to be isocaloric and isonitrogenous and were pelleted fresh and stored in darkness to prevent auto-oxidation of the lipid sources.

Each rabbit was weighed at the beginning and at the end of the trial and received 150 grams of feed per day, to maintain its body weight throughout the experimental period. The consumption of feed was mostly complete and without significant differences between groups. No refusal was observed during the trial. Hazelnut skin supplementation did not lead to palatability problems and this diet was consumed as well as other two diets.

Analytical determinations of diets

The chemical composition of the diet was determined following (34). Diets were analysed to determine dry matter, crude protein, crude ash (by ignition at 550°C), ether extract (using the Soxhlet method), gross energy (by means of an adiabatic bomb calorimeter IKA C7000, Staufen, Germany) and neutral detergent fibre as described by (35). FAs of the diets were determined as their methyl esters. Analysis was carried out by gas chromatography as reported by (36).

Extraction and antioxidant activity of HS

Raw HS derived from roasted hazelnuts were obtained from the Nocciole Marchisio S.p.A. (Cortemilia, CN, Italy) and milled by grinding the HS in a Cyclotec mill (Tecator, Herndon, VA, USA). HS, previously defatted with hexane (1:10 w/v), were then extracted through microwave-assisted extraction (MAE) in a SynthWAVE reactor (Milestone, Bergamo, Italy) with acetone/water 80/20 (v/v %) for 40 min at 60 °C, power (1.5 kW), under nitrogen pressure (5 bar), and at a plant/solvent ratio of 1:10 (w/v). These conditions resulted in the best extraction yield and the greatest phenolic content of HS, as reported elsewhere (37).

The radical scavenging ability of the HS extract was monitored using the stable free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl) according to the UV-Vis colorimetric assay described by (38). A DPPH• solution (0.1 mM) was prepared to give absorbance at 517 nm in the 0.45-0.55 range. The reaction started when 700 µL of the sample diluted solutions were added to a cuvette containing 700 µL of the DPPH• solution. Mixtures were

shaken vigorously and kept in the dark for 20 min at room temperature (time required to reach equilibrium). The UV absorbance of each sample was then measured at 517 nm against a pure methanol blank on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Results are expressed as DPPH• inhibition percentage (I %), which were calculated as follows: $I\ \% = (Abs_{DPPH\cdot} - Abs_{sample} / Abs_{DPPH\cdot}) \times 100$

The bleaching rate of DPPH• was monitored in the presence of different solutions of Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) and HS extract in order to calculate the EC_{50} (amount of extract necessary to decrease the initial concentration of DPPH• to 50% at the equilibrium). Radical scavenging activity in the Trolox solutions was measured at concentrations of around 1, 2, 4, 5, 7, 10, 12, 18 µg/ml. Various concentrations of HS extract between 1 and 500 µg/ml were tested. Measures were performed in triplicate. DPPH• radical scavenging activity is expressed as µg compound/dry extract per ml solution ± standard deviation (SD). Trolox equivalents (µmol/g of extract) were calculated based on the EC_{50} values.

Blood collection

Blood samples were collected from all rabbits at days 1 and 63 and all manipulations were carried out between 09:00 and 16:00 according to the suggestion of the Local Bioethical Committee of the Turin University and all efforts were made to minimize animal discomfort. Approximately 2.5 ml blood was collected from each rabbit via the central ear vein in a quiet, well-lit area to minimize stress. The rabbits were restrained in a towel, with eyes covered. A 22-gauge butterfly fastened to a 2.5 ml syringe was used to collect the blood sample. To avoid haematoma formation, gentle suction was followed by firm pressure after sampling. Blood samples were allowed to clot at 4°C overnight in the refrigerator, and the serum was separated by centrifugation at 3000 × g for 15 min and frozen at –80 °C until analysis.

Measurement of blood serum metabolites

Biochemical analysis was carried out on serum samples. Serum ALT, AST, triglycerides, and cholesterol concentrations were measured by enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex Diagnostics Srl., Florence, Italy).

Measurement of blood serum lysozyme

Serum lysozyme was measured with a lysoplate assay (39) carried out in a moist incubator at 37°C for 18 min. The method is based on the lyses of *Micrococcus lysodeikticus* in 1% agarose. The diameter of the lysed zones was measured with a ruler and compared with the lysed zones of a standard lysozyme preparation (M 3770, Sigma, Milan, Italy). The value is expressed as µg/ml.

Measurement of serum ROM levels

Reactive oxygen metabolites (ROMs) were evaluated using the d-ROMs test (Diacron s.r.l., Grosseto, Italy), as described by (40). The d-ROMs test results are expressed in arbitrary units known as Carratelli Units (CARR U) according to the following formula: $CARR\ U = F(\Delta Abs / min)$

where: F is a correction factor with an assigned value (approximately 9000 at 37°C according to the results obtained with the standard) and (ΔAbs/min) is the mean difference of the absorbances recorded at 1, 2, and 3 min.

Measurement of total serum antioxidant capacity

Serum antioxidants (OXY) were evaluated by the OXY test (Diacron). This test evaluates the capacity of serum to counteract the massive oxidative action of a hypochlorous acid (HClO) solution. Results are expressed in µmol HClO/L according to (41). The balance between oxidative stress and antioxidant capacity was calculated as an oxidative stress index, as described by (42).

Statistical analyses

The EC_{50} and Probit Regressions were computed using an algorithm (43) in Microsoft Visual Basic 6.0 (Microsoft Corporation, Redmond, WA, USA). Statistical analyses of biochemical parameters, serum oxidative status and immune function values were performed using the SPSS software package (version 25 for Windows, SPSS Inc., Chicago, IL, USA). All parameters at day 1 and 63 were compared between groups by one-way analysis of variance (ANOVA) for feeding effects, and means were compared by Tukey post hoc comparisons test. An ANOVA test for multifactorial analysis of variance was used for the two

main factors (diet, time) to identify differences. Time effects (2 levels: day 1 and 63), diet effects (3 levels: LS, LS+HS and PO) as well as time×diet interactions were considered to be statistically significant at $P < 0.05$.

Results and Discussion

Chemical composition and fatty acid profile of the experimental diets

The three diets were formulated to satisfy the nutritional requirements of adult rabbit. The chemical composition and the raw materials of the diets are shown in Table 1.

The most abundant fatty acids (FA) in the LS and LS+HS diets were α-linolenic, linoleic, and oleic acid, accounting for 84.4 to 85.2% of total fatty acid methyl ester (FAME); the most abundant FAs in the PO diet were palmitic, oleic, and stearic acid, which made up 83.1% of total FAME (Table 2). The FA pattern of the three diets was characterized by higher percentages of saturated FAs (45.4 *vs* 13.8 and 13.1% of total FAME) and lower percentages of PUFA (35.2 *vs* 67.2 and 67.6% of total FAME) in the PO diet than in either the LS or the LS+HS diet. Moreover, the n-6/n-3 ratios in the LS and the LS+HS diet were lower (0.46 and 0.50, respectively) than those in the PO diet (3.07) due to its low α-linolenic acid content.

Table. 1 Ingredients and chemical composition of the experimental diets

	Diet/group[1]		
	LS	LS-HS	PO
Ingredients %			
Wheat	39.7	39.1	39.5
Lucerne meal	23.9	23.0	24.3
Wheat straw	27.5	27.5	28.5
Soybean meal	1.55	1.55	1.55
Linseed	5.0	5.0	0
Hazelnut skins	0	1.5	0
Palm oil	0	0	3.8
Dried carob	1.25	1.25	1.25
Yeast	0.058	0.058	0.058
Aminoacids[2]	0.267	0.267	0.267
Minerals[3]	0.25	0.25	0.25
Vitamin-mineral premix[4]	0.525	0.525	0.525
Chemical composition			
Dry matter,%	94.34	94.26	94.32
Crude ash, %	7.90	7.80	7.85
Ether extract, %	2.98	3.01	2.95
Crude protein, %	17.33	17.42	17.41
Neutral detergent fibre, %	31.71	31.73	31.70
Gross energy, MJ/kg	173.9	174.1	173.6

[1] LS=linseed diet; LS+HS=linseed+hazelnut skins diet; PO=palm oil diet; [2] DL-methionine and L-Lysine; [3] Bicalcium phosphate, Calcium carbonate, Sodium chloride and Magnesium oxide; [4] per kg of diet: Sepiolite 212 mg, Vitamin A 12800 IU, Vitamin D_3 1120 IU, Fe 180 mg, Mn 54 mg, Zn 67.5 mg, Cu 11.7 mg, I 0.91 mg, Co 0.63 mg, Cu 0.6 mg

Antioxidant activity of HS

The DPPH• assay was performed to evaluate HS antioxidant activity, as this *in vitro* test is one of the most accurate and responsive methods for analyzing vegetal matrix extracts. Figure 1 shows the DPPH• I% curve which represents Trolox concentration. The DPPH• scavenging activity of HS (expressed as the mean of the EC_{50} values ± SD) was 43.9±6.8 μg/ml,

Table. 2 Fatty acid profile (% total fatty acid methyl ester) of the experimental diets (n=2)

	Diet/group[1]		
	LS	LS+HS	PO
Myristic acid ($C_{14:0}$)	0.10	0.10	1.00
Palmitic acid ($C_{16:0}$)	8.60	8.30	28.60
Stearic acid ($C_{18:0}$)	4.95	4.55	15.80
Arachidic acid ($C_{20:0}$)	0.20	0.20	n.d.
Saturated fatty acids	13.85	13.15	45.40
Palmitoleic acid ($C_{16:1}$)	0.10	0.10	2.60
Oleic acid ($C_{18:1n-9}$)	17.75	23.00	12.20
Vaccenic acid ($C_{18:1n-7}$)	0.80	0.90	4.60
Gondoic acid ($C_{20:1n-9}$)	0.20	0.20	n.d.
Monounsaturated fatty acids	18.85	24.20	19.40
Linoleic acid ($C_{18:2n-6}$)	21.15	20.65	26.55
α-Linolenic acid ($C_{18:3n-3}$)	45.65	41.65	8.65
Stearidonic acid ($C_{18:3n-4}$)	0.55	0.45	n.d.
Polyunsaturated fatty acids (PUFA)	67.35	62.75	35.20
n-6/n-3 PUFA ratio	0.46	0.50	3.07

[1] LS=linseed diet; LS+HS=linseed+hazelnut skins diet; PO=palm oil diet

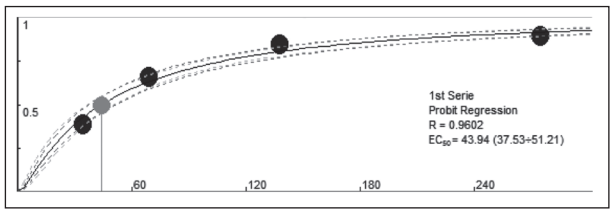

Figure 1. Antiradical activity of the hazelnut skin extracts against the stable free radical DPPH•

and the Trolox equivalent was 358.3±55.8 μmol/g of dry extract. Based on this result, albeit a bit lower than previously reported (37), the HS showed it possessed a good antioxidant capacity for use as a dietary supplement in rabbits.

Reactive oxygen metabolites test, OXY test, and their ratio

Levels of ROMs, OXY, and their ratio, measured at day 1 and day 63, are presented in Table 3. At day 1, no significant differences in oxidative stress and antioxidant status were observed between the three groups, while at day 63 a significant increase (P<0.05) in ROMs was noted for group LS as compared to the other two groups at the same time point and to baseline. At the end of the 9-week trial, OXY was also significantly lower (P<0.05) in the LS group than in either the LS+HS or the PO group. A significant difference (P<0.05) was seen between the LS+HS and the PO group. OXY decreased between day 1 and day 63 in the LS group but increased in the PO group. Furthermore, a significant increase (P<0.05) in the ROMs/OXY ratio was observed for the LS group as compared to the other two groups, indicating a major burden of redox imbalance.

When considering time of sampling as an independent factor, significant differences among experimental groups in terms of redox unbalance were observed for ROMs and ROMs/OXY ratio, while no differences were observed in OXY levels. Moreover, when the different dietary strategy was taken into account, all indices considered resulted significantly influenced (P<0.05). Analysis of the time × diet interaction was significant for ROMs and OXY levels as well as for their ratio (P <0.001).

Recommendations for welfare conditions of intensively kept animals were laid down in a report by (44). However, there is a lack of data about parameters to evaluate acceptable welfare conditions. Different methods have been proposed for the assessment of farmed animal welfare according to zootechnical, clinical, behavioral, hormonal, biochemical and immunological criteria. Clinical chemistry is desirable due to the robustness of the main parameters measured, the reproducibility of the results, and the possibility to evaluate both stress and copying ability (3).

Since the oxidative stress markers and antioxidant parameters measured at day 1 and taken as baseline values were in line with previously published data (45), we assumed that the living and growing conditions of

Table. 3 Reactive oxygen metabolites test (ROMs), OXY test (OXY), their ratio and blood serum metabolites in blood samples (n=8) at day 1 and at day 63

Time, day	1			63			s.e.m.[2]	Time	Diet	Time x Diet
Diet[1]	LS	LS+HS	PO	LS	LS+HS	PO		*P*	*P*	*P*
ROMs, CARR U[#]	298.9	299.7	298.9	463.1[b]	300.3[a]	300.3[a]	2.2	<0.001	<0.001	<0.001
OXY, μmol HClO/l	387.4	388.5	389.0	357.2[a]	390.6[b]	426.1[c]	1.5	0.325	<0.001	<0.001
ROMs/OXY ratio	0.772	0.772	0.769	1.297[b]	0.769[a]	0.705[a]	0.006	<0.001	<0.001	<0.001
ALT, IU/l	33.1	33.2	33.8	33.1	33.5	33.9	1.0	0.948	0.950	0.998
Cholesterol, mg/dl	41.3	41.8	41.5	26.8[a]	29.7[a]	63.4[b]	0.56	0.175	<0.001	<0.001
Triglycerides, mg/dl	71.8	72.7	72.9	46.8[a]	56.0[a]	103.4[b]	0.91	0.043	<0.001	<0.001
AST, IU/l	22.2	22.0	22.4	18.6[a]	19.5[a]	29.1[b]	0.38	0.817	<0.001	<0.001
Lisozyme, μg/ml	16.6	16.1	16.5	26.4[c]	16.4[b]	11.8[a]	0.30	0.005	<0.001	<0.001

[1] LS=linseed diet; LS+HS=linseed+hazelnut skins diet; PO=palm oil diet; [2] s.e.m.= standard error of mean; [#] Reactive oxygen metabolites expressed in arbitrary units known as Carratelli Units (CARR U); [a,b,c] Means with unlike superscript differ (*P*<0.05)

the three groups were able to satisfy their behavioral and physiological needs. A significant change in the capability to deal with oxidative stress was noted with the change in dietary management. Redox imbalance was greatest in the rabbits fed the LS diet, with a significant increase in oxidative stress and a decrease in parameter expression of antioxidant defenses. This phenomenon could be explained by the fact that a diet enriched in PUFA, but without antioxidant protection, could result in increased hepatic lipid peroxidation (46), which may adversely affect hepatoprotection and other recognized benefits related to PUFA supplementation protected with antioxidants.

The present study demonstrated, however, that such negative effects may be counteracted by the concomitant administration of antioxidants, like those contained in the LS+HS diet. As shown in Table 3, this group of rabbits coped better with dietary change, showing minimal changes in ROMs, OXY, and the ROMs/OXY ratio. In the PO group, the FA profile of palm oil (poorer in PUFA and richer in SFA) led to less redox imbalance as compared to the LS group: minor peroxidation of the lipid content occurred as SFA are more stable against peroxidative phenomena.

Blood serum metabolites

The investigation of blood serum metabolites of rabbit represents one of the most valuable tools to monitor feed- and management-related diseases (4). Changes in hematological and biochemical parameters are known to reflect physiological status (45,47).

The blood chemistry results are reported in Table 3. No significant differences in serum metabolites between the three groups were observed at day 1. The values were within the reference range for rabbits (47) and were taken as baseline values.

At day 63, ALT remained unchanged in all three groups, while other serum metabolites showed significant differences ($P<0.05$) among groups.

When considering ALT values neither time, diet nor their interaction were able to determine a significant change in the three groups. Other parameters were not influenced by sampling time, with the exception of triglycerides and lisozyme, which significantly differ ($P<0.05$). Analysis of the diet effect and diet × time interaction were significant ($P <0.05$) for the other serum metabolites.

ALT is the most specific marker of hepatocellular injury and is confined to cytoplasm, whereas AST can be identified to some extent in the heart, skeletal muscles, kidneys, brain, pancreas, and blood cells and is found in both mitochondria and cytoplasm (48). In general, most of the changes in ALT reflect a severe liver disease, although significant diseases in the other above-mentioned organs may also affect its serum levels. Variations in AST, however, are mainly related to initial or minor functional changes and they could also mirror dietary variation in lipid profile (48).

The results of the present study suggested that consuming a diet enriched with palm oil, containing high levels of saturated FAs, could exert a detrimental effect on liver and other several organs, as already determined by other authors (46), while a diet rich in PUFA and antioxidants could have a protective effect. At day 63, cholesterol and triglyceride levels were significantly increased ($P<0.05$) in the PO group as compared to the LS and LS+HS groups, in which a decrease in both cholesterol and triglycerides over baseline was recorded. Elevated levels of plasma triglycerides and cholesterol may reflect lipidemia due to dietary imbalances. The most common cause of this alteration is unquestionably over-feeding. However, the ingestion of more calories than needed not only increases adipose tissue, but also promotes triglyceride synthesis by the liver (49). Different types of FAs differentially affect serum triglyceride levels; in fact, medium-chain saturated FAs have been reported to increase triglyceride levels, while PUFA have been reported to reduce serum triglycerides and cholesterol in some patients with hypertriglyceridemia and hypercholesterolemia (45,49,50), as shown in the present study.

Lysozyme titration is essentially related to the function of the macrophage system and indicates the presence of inflammation. A lack of lysozyme could be correlated with a decreased ability of the immune system to cope with environmental pathogens. Since there was no significant difference in lysozyme values measured at day 1 (Table 3), they were considered as baseline values. They were lower than those reported by (51) for healthy rabbits (27.2 μg/ml), while similar to those observed by (52) in the plasma of male rabbits (10.7 μg/ml). In the rabbits fed the PO diet, serum

lysozyme at day 63 was significantly lower ($P<0.05$) than that of the rabbits fed the LS+HS diet, which was lower again than that of the LS group. The increase in serum lysozyme in the rabbits fed the LS diet (not protected by antioxidants) may indicate the presence of inflammatory conditions, which is correlated with an increase in free radicals. Differently, serum lysozyme was lower at day 63 in the rabbits fed the PO diet as compared to their baseline values because a PO-enriched diet is more stable against peroxidation.

Conclusions

Nutritional interventions can be correlated with changes in serum biochemical parameters related to animal welfare and oxidative status. Diets containing high PUFA levels can have positive effects (decreased levels of cholesterol, triglycerides and AST) as compared to diets containing saturated fats but, unless sufficiently protected with antioxidants, they can cause damage to the body.

We found that the high levels of PUFAs present in linseed, which do not contain antioxidant protection, lead to an increase in free radicals and in lysozyme (related to inflammatory response) and a decrease in antioxidant defenses as compared to the diets containing palm oil, rich in most stable SFA. We also found that hazelnut skins possess a high antioxidant power able to protect the PUFAs present in linseed. HS could find potential use as nutraceutical and dietary supplements in rabbit diets enriched with PUFA, with beneficial effects on serum oxidative status and immune function. In the present study, they exerted a lowering effect on triglyceridemia and cholesterolemia over baseline values.

The use of linseed combined with HS offers the advantage of improving the parameters related to nutritional status, without the risk of inducing high oxidative and inflammatory stress. Moreover, serum biochemical evaluations effectively allow for monitoring animal welfare status, *in vivo*, even before these consequences affect meat products intended for human consumption. Oxidative stress parameters can be useful for the *in vivo* assessment of livestock welfare in farmed animals subject to stressful nutritional treatments or to study how oxidative status in livestock can vary with disease or physiological states such as weaning, pregnancy or lactation.

References

1. Cerioli M, Brivio R, Salogni C, Grilli G, Lavazza A. Valutazione dello stato zoosanitario e immunitario per la individuazione di parametri 'in campo' del benessere del coniglio allevato. Riv Coniglicoltura. 2006; 46(4):18–19.
2. Ludwig N, Gargano M, Luzi F, Carenzi C, Verga M. Applicability of infrared thermography as a non invasive measurement of stress in rabbits. World Rabbit Sci. 2007; 15(4):199–206.
3. Brambilla G, Civitareale C, Ballerini A, et al. Response to oxidative stress as a welfare parameter in swine, Redox Report. 2002; 7(3):159–163.
4. Króliczewska B, Miśta D, Króliczewski J, et al. A new genotype of flax (*Linum usitatissimum* L.) with decreased susceptibility to fat oxidation: consequences to hematological and biochemical profiles of blood indices. J Sci Food Agric. 2017; 97(1):165–171.
5. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC Jr. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatol. 2008; 47(4):1363-70.
6. Lykkesfeldt J, Svendsen O. Oxidants and antioxidants in disease: Oxidative stress in farm animals. Vet J. 2007; 173(3):502–511.
7. Pastorelli G, Rossi R, Cannata S, Corino C. Total antiradical activity in male castrated piglets' blood: Reference values. Ital J Anim Sci. 2010; 8(2s):640–642.
8. Chirase NK, Greene LW, Purdy CW, et al. Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. Am J Vet Res. 2004; 65:860–864.
9. Brambilla G, Ballerini A, Civitareale C, et al. Oxidative stress as a bio-marker of estrogen exposure in healthy veal calves. Anal Chim Acta. 2003; 483(1-2):281–288.
10. Khadija A, Ati A, Mohammed S, Saad AM, Mohamed HE. Response of broiler chicks to dietary monosodium glutamate. Pak Vet J. 2009; 29(4):165–168.
11. Mozaffarian D, Wu JH. (n-3) fatty acids and cardiovascular health: are effects of EPA and DHA shared or complementary? J Nutr. 2012; 142(3):614S–625S.
12. Longato E, Meineri G, Peiretti PG. The effect of *Amaranthus caudatus* supplementation to diets containing linseed oil on oxidative status, blood serum metabolites, growth performance and meat quality characteristics in broilers. Anim Sci Pap Rep. 2017; 35(1):71–86.
13. Longato E, Meineri G, Peiretti PG. Effects of diets containing linseed oil or lard and supplemented with pumpkin seeds on oxidative status, blood serum metabolites, growth performance, and meat quality of naked neck chickens. Can

J Anim Sci. 2018; 98:607–618.
14. Meineri G, Mussa PP, Forneris G, et al. Effects of supplementing with red wine solids on the oxidative status in pigs fed diets with different fatty acid profiles. Large Anim Rev. 2015; 21:251–257.
15. Meineri G, Cornale P, Tassone S, Peiretti PG. Effects of Chia (*Salvia hispanica* L.) seed supplementation on rabbit meat quality, oxidative stability and sensory traits. Ital J Anim Sci. 2010; 9(e10):45–49.
16. Peiretti PG, Meineri G. Effects of golden flaxseed supplementation on the performance and feed digestibility of rabbits. J Anim Vet Adv. 2008; 7(1):56–60.
17. Peiretti PG, Meineri G. Effects of diets with increasing levels of golden flaxseed on carcass characteristics, meat quality and lipid traits of growing rabbits. Ital J Anim Sci. 2010; 9(e70):372–377.
18. Kouba M, Benatmane F, Blochet JE, Mourot J. Effect of a linseed diet on lipid oxidation, fatty acid composition of muscle, perirenal fat, and raw and cooked rabbit meat. Meat Sci. 2008; 80:829–834.
19. Benatmane F, Kouba M, Youyou A, Mourot J. Effect of a linseed diet on lipogenesis, fatty acid composition and stearoyl-CoA-desaturase in rabbits. Animal 2011; 5(12):1993–2000.
20. Dalle Zotte A, Szendro Zs. The role of rabbit meat as functional food. Meat Sci. 2011; 88:319–331.
21. Matics Z, Cullere M, Szín M, et al. Effect of a dietary supplementation with linseed oil and selenium to growing rabbits on their productive performances, carcass traits and fresh and cooked meat quality. J Anim Physiol Anim Nutr. 2017; 101(4):685–693.
22. Menchetti L, Canali C, Castellini C, Boiti C, Brecchia G. The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy. Res Vet Sci. 2018; 118:126–133.
23. Villaverde C, Baucells MD, Cortinas L, Hervera M, Barroeta AC. Chemical composition and energy content of chickens in response to different levels of dietary polyunsaturated fatty acids. Arch Anim Nutr. 2005; 59(4):281–292.
24. Castellini C, Dal Bosco A, Bernardini M, Cyril HW. Effect of dietary vitamin E on the oxidative stability of raw and cooked rabbit meat. Meat Sci. 1998; 50:153–161.
25. Corino C, Pastorelli G, Pantaleo L, Oriani G, Salvatori G. Improvement of color and lipid stability of rabbit meat by dietary supplementation with vitamin E. Meat Sci. 1999; 52:285–289.
26. Dal Bosco A, Castellini C, Bianchi L, Mugnai C. Effect of dietary α-linolenic acid and vitamin E on the fatty acid composition, storage stability and sensory traits of rabbit meat. Meat Sci. 2004; 66:407–413.
27. Tres A, Bou R, Codony R, Guardiola F. Dietary n-6- or n-3-rich vegetable fats and α-tocopheryl acetate: Effects on fatty acid composition and stability of rabbit plasma, liver and meat. Animal. 2009; 3:1408–1419.
28. Rebollar PG, García-García RM, Arias-Álvarez M, et al. Reproductive long-term effects, endocrine response and fatty acid profile of rabbit does fed diets supplemented with n-3 fatty acids. Anim Reprod Sci. 2014; 146(3-4):202–209.
29. Rodríguez M, García-García RM, Arias-Álvarez M, et al. A diet supplemented with n-3 polyunsaturated fatty acids influences the metabolic and endocrine response of rabbit does and their offspring. J Anim Sci. 2017; 95(6):2690–2700.
30. Shahidi F, Alasalvar C, Liyana-Pathirana CM. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. J Agric Food Chem. 2007; 55:1212–1220.
31. Mercanlìgil SM, Arslan P, Alasalvar C, et al. Effects of hazelnut-enriched diet on plasma cholesterol and lipoprotein profiles in hypercholesterolemic adult men. Eur J Clin Nutr. 2007; 61:212–220.
32. Contini M, Baccelloni S, Massantini R, Anelli G. Extraction of natural antioxidants from hazelnuts (*Corylus avellana* L.) shell and peels wastes by long maceration at room temperature. Food Chem. 2008; 110:659–669.
33. Alasalvar C, Shahidi F, Amaral JS, Oliveira BPP. Compositional Characteristics and Health Effects of Hazelnut (*Corylus avellana* L.): An Overview. In: Alasalvar C, Shahidi F, editors. Tree Nuts: Composition, Phytochemicals, and Health Effects. Boca Raton, FL: CRC Press, Taylor and Francis Group; 2009; p. 185–214.
34. AOAC. Official method of analysis of the AOAC. Arlington: AOAC International. 1990.
35. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. J Dairy Sci. 1991; 74:3583–3597.
36. Peiretti PG, Meineri G. Effects on growth performance, carcass characteristics, and the fat and meat fatty acid profile of rabbits fed diets with chia (*Salvia hispanica* L.) seed supplements. Meat Sci. 2008; 80(4):1116–1121.
37. Alexandru L, Binello A, Mantegna S, Boffa L, Chemat F, Cravotto G. Efficient green extraction of polyphenols from post-harvested agro-industry vegetal sources in Piedmont. C R Chim. 2014; 17:212–217.
38. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Tech. 1995; 28:25–30.
39. Osserman EF, Lawlor DP. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J Exp Med. 1966; 124(5):921–952.
40. Benedetti S, Lamorgese A, Piersantelli M, Pagliarani S, Benvenuti F, Canestrari F. Oxidative stress and antioxidant status in patients undergoing prolonged exposure to hyperbaric oxygen. Clin Biochem. 2004; 37:312–317.
41. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Yamamoto T, Morita M. Short-term effects of non-surgical periodontal treatment on plasma level of reactive oxygen metabolites in patients with chronic periodontitis. J Periodontol. 2009; 80:901–906.
42. Castillo C, Pereira V, Abuelo A, et al. Preliminary results in the redox balance in healthy cats: influence of age and

gender. J Feline Med Surg. 2013; 15(4):328–332.
43. Locatelli M, Gindro R, Travaglia F, Coïsson J, Rinaldi M, Arlorio M. Study of the DPPH•-scavenging activity: development of a free software for the correct interpretation of data. Food Chem. 2009; 114:889–897.
44. Verga M. Intensive rabbit breeding and welfare: development of research, trends and applications. In: Berchiche M, Zerrouki N, Lebas F, editors. World Rabbit Science Association. Proceedings of the 7th World Rabbit Congress, 4-7 July; Valencia, Spain: Vol. B, 2000; p. 491–506.
45. Meineri G, Giacobini M, Forneris G. Evaluation of physiological parameters of the plasma oxidative status in rabbits. J Appl Anim Res. 2017; 45:315–319.
46. Xu J, Rong S, Gao H, et al. A combination of flaxseed oil and astaxanthin improves hepatic lipid accumulation and reduces oxidative stress in high fat-diet fed rats. Nutrients. 2017; 9:271.
47. Kaneko JJ, Harvey JW, Bruss ML. Clinical biochemistry of domestic animals. 6th ed. Amsterdam: Academic Press. 2008.
48. Ravel R. Clinical Laboratory Medicine: Clinical Application of Laboratory Data. JAMA. 1995; 274:1088–1089.
49. Scott D, Kurenitz M. Using lipid-lowering agents effectively. When diet is not enough. Postgrad Med. 1990; 87(8):171–186.
50. Rodriguez M, Carro MD, Valiente V, Formoso-Rafferty N, Rebollar PG. Effects of dietary fish oil supplementation on performance, meat quality, and cecal fermentation of growing rabbits. J Anim Sci. 2017; 95(8):3620–3630.
51. Moscati L, Dal Bosco A, Battistacci L, Cardinali R, Mugnai C, Castellini C. Native immunity and oxidative traits of growing rabbits. World Rabbit Sci. 2008; 16:213–220.
52. Bonnafous R, Raynaud P. Le lysozime dans le matériel et les secrection digestives chez le lapin domestique. Repr Nutr Dev. 1980; 4:1273–1278.

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